Development of HPLC method for phenylethyl resorcinol content determination in whitening cream preparation

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ABSTRACT: This experiment aimed to develop and validate an analytical method using HPLC for phenylethyl resorcinol (PR) content determination in the emulsion-cream formula, consisted five preservative agents methylparaben, ethylparaben, propylparaben, butylparaben, and phenoxyethanol. Measurement was carried out with a UV detector and using a C18 column.   Previously, a selectivity test was conducted to distinguish PR from the other whitening agents that were hydroquinone, alpha-arbutin, kojic acid, and vitamin C. The mobile phase system was optimized by using acetonitrile, water, and methanol, in a gradient concentration. The optimum condition was set in the flow rate of 1.0 mL/min, which provided a suitable chromatogram. Linearity in the range of 10.48-73.36 mg/mL showed a correlation coefficient (r) = 0.9999 and generated the detection and quantification limit of 0.7183 mg/L and 2.3943 mg/L, respectively. A robustness test was performed by a variate internal and external factor changes also proved the proper precision. Finally, the validated method was applied as an adequate quantitative analysis to determining phenyl ethyl resorcinol content in the whitening cream preparations.

KEYWORDS: Phenyl ethyl resorcinol; preservatives; whitening cream; HPLC; reverse phase; gradient mobile phase; validation method.

1. INTRODUCTION

Perception of beauty is changing from time to time and depends on the origin of the person who perceives it, concurrently by the growth of the fashion daily. Then, the appearance treatments are no longer just a desire but have become a necessity. Especially in Asia, "white" skin is an important parameter to be beautiful. A preference for fair women grows into the ideal that is exploited by cosmetic manufacturers [1-2]. It proved that public perception about the fairness of skin is one barometer of female beauty and cannot be separated from the advertisement of cosmetic products [3-4]. Products that promise to improve the tone and brightness, ranging from make-up to the body care, all with frills to whitening-skin, have the highest sales figures in ASEAN countries [5].

Nowadays, both natural and synthetic substances have been used as the whitening agent. Some materials from the natural resources are strongly assumed by people as safer to human skin compared to the synthetic-chemical. Examples of natural skin whitening ingredients are kojic acid, arbutin, licorice chamomiles, mulberry, and green tea extract. Moreover, these are added by the newest generation of the whitening agent [6,7], namely phenylethyl resorcinol (PR), a phenol derivative. This compound is used to brighten the skin by fighting the process of melanin production. Compared to other lightening agents such as kojic acid and arbutin, PR was found to possess superior performance [8].

Even organic living matters, it still has the potential to bring a lousy impact if it has been used excessively [1, 9-11]. However, to obtain maximum results in a relatively fast time, some cosmetic industries tend to use PR in exceeds 0.5% w/w. There were several cases of preparation found. It will impact to cause skin allergies if applied over the permitted. On August 7, 2012, the China State Food and Drug Administration (SFDA) approved PR (SymWhite® 377) as a skin whitening ingredient in skincare cosmetics with a 0.5% usage limit [12,13].

An *in vitro* absorption study of PR using an HPLC method has been carried out in Thailand by Limsuwan *et al.* [14] and in China by Li *et al.* [15]. However, in the framework of monitoring, there is no

standard method to analyze this substance's level in cosmetic preparations. Whereas, a cosmetic product is one of the most complex preparations due to many ingredients consisted besides the primary functional matter. Even some method developments for phenyl ethyl resorcinol have been reported, the specificity from other whitening agents and the common polar preservative agents used in cream preparation had never been conducted concurrently in the content determination. Commonly, the HPLC methods using the isocratic system supported by a UV spectrophotometry detector [14-18]. In this experiment, a specific content determination for PR in cream preparation consisted of other whitening agents and preservatives was developed using a gradient mobile system which never been reported before.

Chemically, PR $C_{14}H_{14}O_2$ (4-(1-phenylethyl)benzene-1,3-diol) is a phenol derivative with pKa 9.46, soluble in ethanol, and insoluble in water. It has activity as inhibitor tyrosinase [8, 14, 19]. Based on its structure in Figure 1 and polarity, it is needed a reverse phase HPLC development to separate and measure its content appropriately.
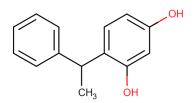

Figure 1. Structure of PR [20].

A whitening cream in the market usually consists of several whitening agents in one preparation to modulate the effect. Besides, cosmetic preparation also always contains some preservatives due to its multiple applications, which led to microbial contamination. Parabens are the common preservatives agents which widely used, besides phenoxyethanol. These were intentionally added to samples of facial whitening creams. Therefore, a suitable method should be developed to measure PR concentration, which is specific, accurate, precise, and robust.

In this experiment, an HPLC method was developed by optimized the mobile system using a gradient-mobile phase system and tested the specificity of PR from the other kinds of whitening agent, that were hydroquinone, alpha-arbutin, kojic acid, and vitamin C. Next, a content determination of PR in the formula consisted five common preservatives, that were methylparaben, ethylparaben, propylparaben, butylparaben, and phenoxyethanol was developed and validated. Due to the polarity of PR and the preservatives, a reverse-phase column using a gradient mobile system was considered to produce a suitable method. Finally, the validated method was applied to determine PR content in the typical cream formulation and marketed product.

2. RESULTS

2.1. System optimization

The first step of HPLC method development is optimizing the systems involved wavelength screening, mobile phase, rate of injection, and used the results to check its suitability [20-24]. In this study, PR's response will be distinguished from the four other whitening compounds, that were hydroquinone, alpha-arbutin, kojic acid, and vitamin C. Next, a content determination of PR in the formula contained five common preservatives, that were methylparaben, ethylparaben, propylparaben, butylparaben, and phenoxyethanol was developed and validated. The HPLC method must be tested to meet the suitability requirements as well as fulfills the validation test parameters, including the robustness evaluation [20-25, 27-29]. Therefore, system optimization was performed thoroughly, continued by the validation test.

2.1.1. Wavelength optimization

In this method, the analyte in the mixture was dissolved with methanol [14], which showed better solubility compared using the other solvent, which was examined. Screening of UV absorption was conducted by observing the maximum wavelength of PR, which found at 282 nm. Then, the response of all preservatives also was checked in this wavelenght and resulted in the spectra, as shown in Figure 2. This figure shows the wavelengths data as follows: methylparaben (257 nm), ethylparaben (λmax = 257 nm), propylparaben (257

nm), butylparaben (257 nm), phenoxyethanol (271 nm), respectively. Basically, the λ-max of PR alone was tested before and existed at 280 nm, which is similar to other reports [13, 15, 17].
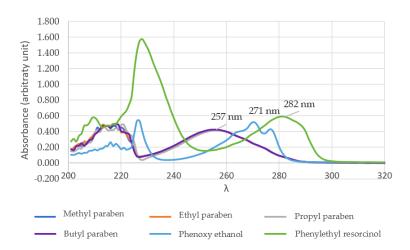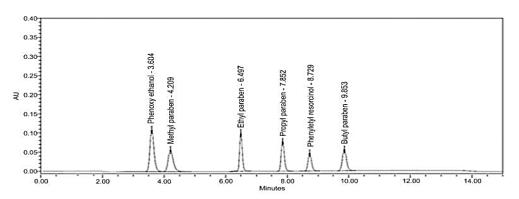

Figure 2. The UV spectra of PR and the five preservatives.

2.1.2. Mobile phase optimization

A column C18 from Zorbax (USA) with particle size 5 µm/4.6 x 150 mm was selected to be used in this method involved in an HPLC Waters e2695 (USA). Table 1 revealed the result of mobile phase optimization, which involved acetonitrile, methanol, and water, in a series of concentration.

Table 1. Mobile phase variations used in optimization.

No	Mobile Phase	Results
a.	Acetonitrile-methanol-water (40:20:40)	Only five compounds have been separated well.
b.	Acetonitrile-methanol-water (50:20:30)	Only four compounds have been separated well.
c.	Acetonitrile-methanol-water (30:20:50)	Only four compounds have been separated well.
d.	Acetonitrile-methanol-water (35:5:60)	Only four compounds have been separated well.
e.	Acetonitrile-water (30:70)	Only three compounds have been separated well.
f.	Min 0-4: Acetonitrile-water (30:70) Min 4-15: Acetonitrile-methanol-water (40:20:40)	Six compounds have been separated well, included PR.

Figure 3 reveals the chromatogram from the last system, that was separated into two steps: 0-4 min using acetonitrile-water (30:7) followed by acetonitrile-methanol-water (40:20:40) during 4-15 min.
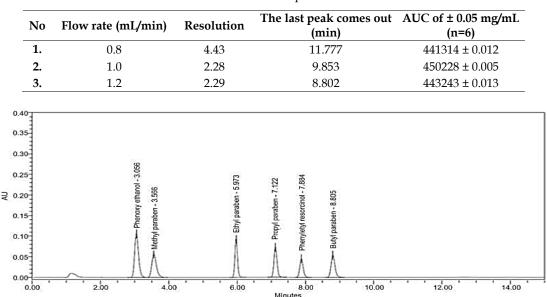

Figure 3. Chromatogram of the optimized system: in 0-4 min and acetonitrile- water (30:70) continuing by acetonitrile-methanol-water (40:20: 40).

2.1.3. Flow rate optimization

After the appropriate mobile phase was found, then the flow rate was optimized to obtain the best spectra. Table 2 reveals that the flow rate of 0.8 mL/min has the highest resolution, 4.43, and the retention time =11.77 min.

Rate of injection optimization resulted chromatogram as shown in Figure 4.

Table 2. Flow rate optimization.

No	Flow rate (mL/min)	Resolution	The last peak comes out (min)	AUC of ± 0.05 mg/mL (n=6)
1.	0.8	4.43	11.777	441314 ± 0.012
2.	1.0	2.28	9.853	450228 ± 0.005
3.	1.2	2.29	8.802	443243 ± 0.013

Figure 4. Chromatogram of the optimal flow rate of 1.0 mL/min, 20 µL volume sample.

2.1.4. System suitability test

A system suitability test was conducted to check the effectiveness of the chromatographic system by evaluating some parameters, that were Relative Standard Deviation (RSD) of the area under the curve (AUC), tailing factors, theoretical plats, and capacity factors [20-25]. As the reference, we compile the requirement for an HPLC system[20-25] is depicted in Table 3 with the calculation method for measuring each parameter is explained in Methods (*5.3.5. Suitability System Test*). Hereafter, the complete optimum condition for measuring PR concentration using the gradient mobile system of HPLC was reviewed in Table 4.

Table 3. HPLC system suitability test results.

Parameter [22-25]	Limit of acceptance	Measurement results
(%) RSD area under the curve (AUC)	≤ 2%	0.16 ± 0.002
(%) RSD of retention time	≤ 1%	0.31 ± 0.018
Tailing factors	0.9 – 1.4	1.13 ± 0.22
Theoretical plats	N>2000	26.109 ± 5.042
Capacity factors	k>2	3.88 ± 1.02
Resolution	R_s > 1.5	2.28 ± 0.081
Peak Asymmetry Ratio	≤ 1.5	0.98 ± 0.014

Noted: n=6; RSD: relative of standard deviation, AUC: area under the curve

2.2. Validation Method

The validation method must be performed following the guidelines, such as USP, ICH, and AOAC standard. Specificity, linearity, the range of measurement, the limit of detection, the limit of quantification, accuracy, and precision parameters are tested and referred the compendium and some technical guidelines [20-30], which each also are stated in the Methods part thoroughly. Here is the compilation of the requirements that are depicted in Table 5.

Table 4. Chromatographic conditions for quantitative determination of phenylethyl resorcinol.

Parameter	System
HPLC instrument	HPLC Waters e2695 (USA)
HPLC column	Zorbax Eclipse Plus C18 column particle size 5 μm/4.6 x 150 mm (USA)
Injection volume	20 μL
Flow rate	1.0 mL/min
Mobile phase	Min 0-4: Acetonitrile-water (30:70) Min 4-15: Acetonitrile-methanol-water (40:20:40) Solvents from Merck-Sigma (Germany)
Wavelength	282 nm

Table 5. Validation parameter, limits, and calculation.

Parameter	The limit requirement, experimental, and calculation [20-30]
Specificity	The acceptability criterion was determined by seeing if it no disturbance at the retention time to the main analyte, with the resolution is > 1.5 [23-25].
Linearity	The linearity of 6 concentrations of 20 - 120% from the targeted level must have r ≥ 0.999; with %y- intercept < 2,0% and Vxo<2,0 %[23-25]. The calculation of r is explained in *5.3.6. Validation Methods, Equation 8.*
Range	The range is determined from the limit of quantification (LOQ) to the highest concentration, which yields the RSD < 15%. LOQ can be generated from linear regression [23-25].
Limit of detection (LOD)	Limit of detection is the concentration that shows approximately three times higher than the noise, can be generated from the regression equation: $LOD = 3.3 \left(\frac{Syx}{b}\right)$ (Equation 1). Syx is the standard deviation of the response and b is the slope of the calibration curve [20-30].
Limit of Quantification (LOQ)	Limit of quantification is the concentration, which shows approximately ten times higher than of noise, can be generated from the regression equation: $LOD = 10 \left(\frac{Syx}{b}\right)$. (Equation 2). Theoretically, the Relative Standard Deviation (RSD) of the six replicate-measurements on this concentration must not over 20% [23-25].
Accuracy	Accuracy refers to the difference between the mean of the set of results and the most probable value for the quantity measured or the difference between the result (or) mean and the true value. Normally, the 3 times repetitions result the RSD ≤ 2% and % recovery 98.0 – 102.0% [22-25]. However, considering the influence of concentration level, Howart stated the HorRat value ≤ 2% [26]. The The Howart's RSD is calculated by RSD = ± $2^{(1-0,5\log C)}$, so for 100% level, this value will be = $2^{(1-0,5\log 1)}$ = 2%. After that HorRat ratio calculated by dividing the RSD observation to Horwitz's calculation. HorRat = RSD observed/RSD calculated [26].
Pre cision	Six-time repetitions on the same concentration or the three concentrations of 70 – 120% probable concentration, which were measured in triplicate, must result the value of RSD ≤ 2,0% [23-25].
Robustness	Robustness is defined by the check of the capability of an analytical method to be stable by the small deliberate changes in method conditions. The variable robustness in the HPLC technique may involve internal factors, such as flow rate, λmax shifting, and external factor, for example, operator, column, and material vendor [21-29].

2.2.1. Specificity

Specificity is the ability of a method to measure analytes in the presence of other components, including decomposition results, contamination, similar compounds, and other foreign compounds [23-25]. In this experiment, five preservatives and four common kinds of other whitening agents were assumed to exist in the matrice. The preservatives were the four paraben compounds (methyl, ethyl, propyl, and butylparaben) and phenoxyethanol. Other whitening agents for the specificity test were namely alpha-arbutin, hydroquinone, kojic acid, and vitamin C. Firstly; specificity test was performed by eluted these samples, respectively: the solvents, matrice solution, standard PR solution and obtained the chromatograms which are shown in Figure 5.

Afterward, the single standard solution of the five preservatives and the mixture of PR with other whitening agents were measured, respectively. The complete chromatograms resulted from the specificity test were listed in Table 6 and represented in *Supplementary 1*, which shows no peaks from the other components interfered with PR chromatogram. The data indicated that the analytical method could demonstrate the single signals of PR correctly and selectively. The resolution of each peak of the mixed standard matrice was more than 1.5 as the limit of its acceptability [23-25]. Thereof, this analysis method was proven to be specific for the determination of PR levels in the existence of 5 preservatives as well as can separate PR from the other whitening agents.

Table 6. Specificity test.

No	Sample	Retention time (min)	Resolution	Tailing factor
1.	Solvent	-	-	-
2.	Cream matrice	-	-	-
3.	Gel matrice	-	-	-
4.	A single standard of PR	8.640	-	1.12
5.	A single standard of methylparaben	4.132	-	1.27
6.	A single standard of ethylparaben	6.469	-	1.16
7.	Single standard ot propylparaben	7.780	-	1.15
8.	A single standard of butylparaben	9.741	-	1.18
9.	A single standard of phenoxyethanol	3.581	-	1.19
10.	Mixed standard:			
	PR	8.639	4.57	1.12
	Methylparaben	4.137	2.32	1.24
	Ethylparaben	6.472	11.46	1.12
	Propylparaben	7.797	8.28	1.12
	Butylparaben	9.735	5.24	1.13
	Phenoxy ethanol	3.579	-	1.19
11.	A single standard of alpha-arbutin	1.385	-	1.14
12.	A single standard of hydroquinone	1.624	-	1.09
13.	A single standard of kojic acid	1.467	-	1.43
14.	A single standard of vitamin C	1.304	-	1.59
15.	Mixed standard :			
	PR	8.662	3.05	1.15
	Alpha-arbutin	1.410	-	1.15
16.	Mixed standard :			
	PR	8.650	3.13	1.17
	Hydroquinone	1.632	-	1.10
17.	Mixed standard :			
	PR	8.671	3.09	1.12
	Kojic acid	1.456	-	1.47
18.	Mixed standard :			
	PR	8.669	3.10	1.14
	Vitamin C	1.311	-	1.53

2.2.2. Linearity and range of analysis

The testing was carried out by using seven concentrations, from 20% to 140% w/w. The data from the linearity test was then plotted in a calibration curve, which resulted in the equation: y= 8175.3x - 2709.6. It has a coefficient correlation r = 0.9999 and coefficient variance Vx_0 = 1.39 (each concentration was measured 3x). Therefore, fulfilled the specified requirements, because of the r ≥ 0.999 and Vx_0 <5.0% [23-25]. The data of the linearity test involved limit of detection and limit of quantification is attached in *Supplementary 2*.

2.2.3. The limits of detection and quantification

Limit of detection (LOD) and limit of quantitation (LOQ) represent the sensitivity performance. The values were generated from the linear regression equation following this formula:

$$LOD = 3.3 \left(\frac{Syx}{b} \right) \hspace{4cm} \text{[Eq. 1]}$$

$$LOQ = 10 \left(\frac{Syx}{b} \right) \hspace{4cm} \text{[Eq. 2]}$$

Note: Syx is the standard deviation of the response and b is the slope of the calibration curve.

From the data analysis in *Supplementary 2*, it was found that Syx = 1975.82 and b = 8175.3. Therefore, the final calculation resulted in LOD, and LOQ was 0.7300 mg/L and 2.4300 mg/L, respectively. Some references stated that LOD and LOQ are enough to be generated from the calculation using this mathematical equation due it has been accepted as the general statement [23-25]. However, an experimental measurement was tested by injecting a mixture solution concentration of 0.7300 mg/mL and resulted in the chromatogram, as shown in Figure 6 below. All peaks have still existed in this lowest concentration; however, the noises will be influenced in the measurement. Hence, for a quantitative method, the 3.3 fold of LOD concentration, namely LOQ, should be prepared [22-25].
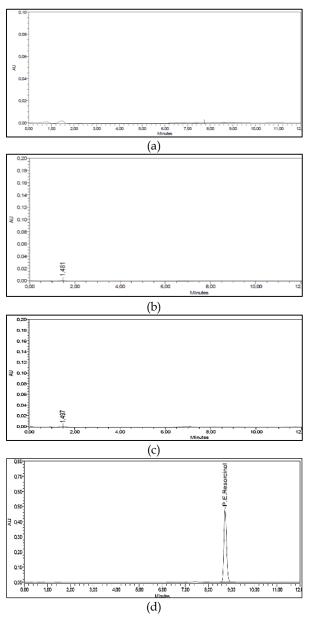

(a)

(b)

(c)

(d)

Figure 5. Chromatograms of solvent (a), cream matrices (b), gel matrices (c), and PR (d).
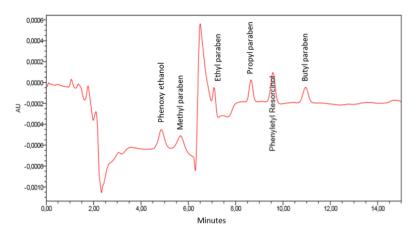
Figure 6. Peaks in the LOD concentration.

2.2.4. Accuracy test

The next validation parameter that must be evaluated was the accuracy with the data depicted in Table 7. It was done by adding raw material to the sample matrices in a concentration range of 80%, 100%, and 120% of PR dose ± 50 mg/L (maximum limit requirement level = 0.5% w/w) [12]. From the analysis, accuracy results represented the highest level of 101.79 and the lowest level of 98.15%. In addition, according to the limit of requirement, this value has fulfilled acceptance criteria [23-26].

Table 7. Accuracy test results.

No	Concentration	Measured level (mg/L)	Theoretical level (mg/L)	% Recovery
1	80%	42.0729		99.98
		41.3019	42.08	98.15
		42.0107		99.84
2	100%	53.0884		100.93
		53.0497	52.60	100.86
		52.2247		99.29
3	120%	61.3079		100.57
		60.4171	60.96	99.11
		62.0515		101.79
	Average (%)			100.06
	Highest recovery			101.79
	Lowest recovery			98.15
	SD			1.110
	RSD (%)			1.110

2.2.5. Precision test

The last parameter of validation was intra-day and inter-day precision, the level of conformity of repeated measurement results of the same analyte solution in the similar measurement conditions. The data tables are depicted in *Supplementary 3* due to the limitation of the table amount, which can be presented. In the table, it was shown that from 6 repetitions, the RSD was 1.5- 1.83 or less than 2%, so it met the requirement [23-25]. Accuracy criteria depend on the concentration of the analyte in the sample; the smaller the analyte, the higher of the RSD. Horwitz equation describes the increase of standard deviation compared to the decreasing of concentration, that formulated as RSD = $2^{(1-0.5 \log C)}$ [26]. RSD value of the observation was divided with the result of Horwitz's calculation yielded the Horwitz Ratio or HorRat. The values are presented in the table of *Supplementary 3*, with the value range between 0.48 to 0.57. All data from intra-day and inter-

day precision testing revealed the RSD < 2% [23-25] and HorRat value <2% [26]. This fact indicates that the analysis method has adequate precision.

2.2.6. Robustness test

As has been explained in the subsequence flow rate optimization, the suitability test parameter, which also represented the robustness, was checked by changing the injection rate from 0.08 to 0.12 mL/min. Table 2 showed that the flow rate of 0.8 mL/min has the best resolution. However, the flow rate of 1.0 mL/min and 1.2 mL/min produces almost the same resolution. The last peak appearance time showed less of one-min distance but resulted in a similar value of area under the curve for an equal solution sample tested.

Secondly, robustness testing was performed by measuring PR in the cream mixture at the different wavelengths, which was 280 nm and 282 nm with the same instrument. The results from seven replications showed that there was no significant difference in AUC resulted between the two wavelengths. The statistical analysis yielded t-calculation = 10.312. This is less if referred to the t-table = 2.18, so it can be stated that the results of measurements at λ=280 nm are not significantly different from the measurement results at λ=282 nm [31,32]. These robustness test data can be seen in *Supplementary 4.* The measurement resulted in %-recovery 99.51 – 100.82%, hence the analysis at λ=280 nm met the precision requirement, besides it was not significantly different from the first condition of the developed method.

In addition, the robustness test was conducted by changing the column and solvent vendor. This test was performed under three variated conditions. The first condition used the same column (Zorbax's) but using the different solvents vendor (Jt. Baker, USA). The second condition employed a different brand column (Enduro, 5μm, 4.0 x 150 mm, Japan), next will be mentioned briefly as the Enduro column, combined with the Merck-Sigma solvents, and the third condition utilized Enduro column and Jt. Baker's solvents. These replications for completing the robustness data were operated by a different person.

Furthermore, the seven times repetition measurement of 0.05 mg/mL PR in the mixture with 0.05 mg/mL parabens and 0.02 mL/mL of phenoxyethanol solution obtained in this experiment are presented in Table 8. This table shows the results of seven replicates measurement on the ± 0.05 mg/mL sample solution under the three conditions. This table shows that the parameter of %-recovery, retention time, resolution, and tailing factor from the three variation-conditions fulfilled the requirements [20-25]. Hence, it can be concluded that the system was stable and robust [27-29].

Table 8. Robustness test data by changing the column and solvent vendor.

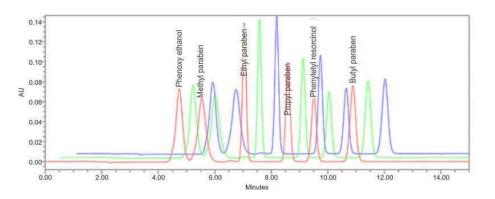| Sample	% Recovery (limit: 98-102%) [23-25]			Retention time (min) (RSD of Rt ≤ 1%) [23, 24]			Resolution (Rs>1.5) [23,24]			Tailing factor (= 0.9-1.4) [23, 24]		
	I	II	III	I	II	III	I	II	III	I	II	III
1	100.03	99.22	99.48	9.854	10.458	9.452	2.781	2.855	2.846	1.11	1.12	1.08
2	100.17	99.98	98.99	9.849	10.483	9.509	2.829	2.848	2.886	1.12	1.09	0.99
3	99.29	99.55	99.78	9.885	10.609	9.388	2.775	2.815	2.870	1.18	1.14	1.09
4	100.02	100.34	100.12	9.805	10.662	9.557	2.905	2.866	2.868	1.14	1.18	1.02
5	100.66	100.47	99.09	9.871	10.508	9.566	2.860	2.809	2.862	1.22	1.09	1.04
6	99.89	100.61	100.02	9.865	10.390	9.421	2.789	2.834	2.854	1.23	1.12	1.05
7	100.55	99.88	100.22	9.822	10.521	9.411	2.822	2.807	2.888	1.09	1.25	1.14
Average	100.09	100.01	99.67	9.85	10.52	9.472	2.82	2.83	2.87	1.16	1.14	1.06
SD	0.45	0.51	0.50	0.03	0.09	0.072	0.05	0.02	0.02	0.06	0.06	0.05
RSD (%)	0.45	0.51	0.50	0.28	0.87	0.76						

Note: I, II, III represent the condition.
I: Column Zorbax, solvents from Jt. Baker
II: Column Enduro, solvent from Merck-Sigma
III: Column Enduro, solvent from Jt. Baker

The chromatograms resulted are depicted in Figure 7 below, which represents similar profiles. All chromatograms show the appropriate retention time, sharp peaks, and in a glance, demonstrate excellent resolutions.

Figure 7. Chromatograms from the measurement: using the column Zorbax with mobile phase from Jt. Baker (red chromatogram), using column Enduro with mobile phase from Jt. Baker (green chromatogram), using column Enduro with a mobile phase from Jt. Baker (blue chromatogram).

2.3. PR determination on whitening products from the market

Based on all results, it can be seen that the analysis method has met the requirement of the validation parameters. The method was then used to determine the level of products circulating in the market. In Indonesia, the requirements for using PR have not been stated for cosmetic products. Meanwhile, on August 7, the Chinese Food and Drug Administration approved PR as a skin whitening ingredient in skincare cosmetics with a 0.5% w/w usage limit [12]. The results of the level determination can be seen in Table 9.

Table 9. Results of determining the levels of facial whitening products in the market.

Sample	Compounds	Concentration (% w/w)	Average ± SD (% w/w)
Cream 1	PR	0.4229 0.4227 0.4212	0.4223 ± 0.001
Cream 2	PR	0.5842 0.5858 0.5849	0.5850 ± 0.001
Cream 3	PR	0.5501 0.5858 0.5849	0.5736 ± 0.020

Note: PR = Phenylethyl resorcinol.

3. DISCUSSION

3.1. System optimization

Some steps have been conducted to optimize the system. Firstly, Figure 2 depicted the PR's λ-max wavelength is 282 nm, it means that the polar preservatives shift the peak 2 nm. At this point, the paraben's spectrums are shown overlapped each other due to their similar chromophore. Another kind of preservative, namely phenoxyethanol, revealed a different profile; however, it is also overlapped with the paraben's and PR's peak. Hence, a suitable chromatographic system was needed to be developed and validated for determining PR's content precisely and accurately.

As has been explained, the solution test was composed of PR added by five preservatives, which totally were polar; therefore, the developed method was optimized using a reverse-phase chromatography. A series of mobile phase were optimized. Firstly, the mobile phase consisted of acetonitrile, water, and methanol

(40:20:40) was attempted following to Lismuwan *et al.* (2017). Nevertheless, the trial showed that it could not separate the six ingredients (PR, ethylparaben, methylparaben, propylparaben, butylparaben, and phenoxyethanol), finely. This different result because of the different ingredient between this mixture to the Lismuwan's preparation and the different of λmax used [14]. Thereof, the variations of the mobile phase were next tried to involve several solvent compositions, as shown in Table 1. This table reveals that the gradient mobile phase (number f) was the best, because it separated all of six test compounds finely, and produced the clear peaks. This appropriate method was performed in two steps of the gradient system. The first promptly used only acetonitrile-water (30:70) from 0 to 4 min, which then was continued by the second step using acetonitrile-methanol-water (40:20:40) from 4.1 to 15 min. As noted, the volume of injection has been tried using 10 and 20 µL, and the 20 µL of sample-solution volume showed a better result with the clear peaks and appropriate reproducibility of AUC.

Figure 3 reveals that the mobile system separated PR from the preservatives clearly with a proper resolution. Besides, the maximum retention time is shown before 10 min. No chromatogram was overlapped. Hence, actually, this result showed that the mobile system could be employed for the quantitative-analyzing of PR and all preservatives simultaneously. However, in this article, our discussion is focusing on the development and validation of the content determination method for PR only. Hereafter, the preservatives were assumed as the matrices for specific performance supported data.

In the flow rate optimization, Table 2 showed that the flow rate of 1.0 mL/min and 1.2 mL/min produces almost the same resolution of 2.2. Figure 4 revealed that the last peak was also not much different, with the only one-min distance. However, the flow rate of 1.2 mL/min will make the HPLC pump work harder. Hence, the optimal flow rate was set at 1.0 mL/min. Its resolution value was obtained 2.28, which it means has fulfilled the requirement (> 1.5) [23-25], with a relatively short test time (the last peak came out after 9.853 min).

Finally, the suitability test was performed to evaluate the acceptability of the developed method which showed in Table 3. This table shows that the HPLC system used has been in a suitable condition that met the requirements and can be tested further on its validation parameters before applicated. The complete of measurement condition was then listed in Table 4.

3.2. Validation Method

Based on the optimum condition of the HPLC system, a validation method was carried out to investigate the consistency based on the standard requirement as shown in Table 5. Validation parameters, namely specificity, linearity, range, limit of detection, limit of quantification, accuracy and precicion tests have been explained thoroughly in the Results sequence. All of results have been referred to the standard requirements as mentioned in each parameter presented, which showed that this gradient mobile system is suitable and adequate.

Nowadays, robustness and ruggedness were used to one term that is robustness. This test must be carried out to ensuring the stability of the method due to the disturbance existences, internal or externally [27-29]. The first robustness test has been performed to check the influence of its internal factor on the result that was the injection rate optimization concurrently. In addition, the robustness test which evaluated the stability of the system toward the change of column and solvent's vendor. As revealed in Table 8, the developed methods was proven to be stable and robust.

As the highlights, the range of measurement which was determined by observing the interval between the highest and lowest levels of the analyte, with the acceptable levels of accuracy, precision, and linearity was obtained as 10.48 – 73.36 mg/L. Next, the final calculation resulted in LOD, and LOQ was 0.7300 mg/L and 2.4300 mg/L, respectively. This result represent the high sensitivity of the methods.

3.3. PR determination on whitening products from the market

In purpose to evaluate the reliability, the optimized method was applied for determining PR content in several marketed whitening creams. Table 9 revealed that all samples of whitening cream contain PR. From the data, it can be seen that two products, cream 2 and 3, containing PR above 0.5% w/w. If referring to the product specification, Chinese Food and Drug Administration, these creams did not meet the requirements.

All data also reveal in a glance that actually, this method can measure the level of the preservatives concurrently. Due to the clear response of each compound, this developed method can be strongly predicted as the specific and sensitive method for quantifying all components in one step simultaneously. However, here, we only focused on PR as one of the new entities used in the cosmetic area, which is not be guided yet,

how to be determined, qualitative, and quantitatively. However, it is still can be developed further to determine all components simultaneously.

4. CONCLUSION

An adequate quantitative analytical for PR in cosmetic cream with five preservatives, namely methylparaben, ethylparaben, propylparaben, butylparaben, and phenoxyethanol, has been developed and validated. This HPLC system equipped by a UV detector at 282 nm, using a C18 column, with a gradient mobile system involved acetonitrile-water in the first step and acetonitrile-methanol-water in the second step. It also can separate PR form the other whitening agents, namely kojic acid, alfa-arbutin, hydroquinone, and vitamin C individually. The method has been proven to fulfill all validation parameter requirements and revealed a robust chromatography system and has been shown as an adequate method for PR content determination in the marketed-whitening cream preparations.

5. MATERIALS AND METHODS

5.1. Materials

The materials were the standard reference of PR (Hangzhou Lingeba Technology, China); cream samples from the market containing PR (Bandung, Indonesia); solvents for mobile phase preparation: acetonitrile (HPLC grade), methanol (HPLC grade) from Merck-Sigma (Germany) and Jt. Baker (USA), aqua-bidestilata (Bandung Institute of Technology); H_2SO_4 (Merck-Sigma, Bandung) the standard of hydroquinone, alpha-arbutin, kojic acid, vitamin C, phenoxyethanol, methylparaben, ethylparaben, propylparaben, butylparaben from Directorate of Food and Drug Monitoring Laboratory Certificated Standard Reference Institution, Jakarta, Indonesia; and the component of cream base: stearic acid, cetyl alcohol, triethanolamine (TEA), propylene glycol, isopropyl myristate, glycerin, disodium EDTA (Merck-Sigma, Bandung).

5.2. Instruments

The instruments used in this study include HPLC Waters e2695 UV/VIS detector (USA), Zorbax Eclipse Plus C18 column particle size 5 µm, 4.6 x 150 mm (USA), Enduro C18 column particle size 5 µm, 4.0 x 150 mm (Japan), Hewlett Packard 8453 spectrophotometer (USA), Wiseclean ultrasonicator (USA), vortex Jeio Tech (USA), Mettler Toledo AG104 analytical scales (Switzerland), 0.20 µm membrane filters, micropipettes, vacuum pumps, glassware such as beaker glass, volumetric flasks, and volume pipettes.

5.3. Procedures

5.3.1. Preparation of standard solutions

a. Master Standard 1

This standard was prepared from a mixture of alpha-arbutin, hydroquinone, kojic acid, and vitamin C standard; each was in a concentration of 0.5 mg/mL using methanol as the solvent.

b. Master Standard 2

A standard solution was made from PR, methylparaben, ethylparaben, propylparaben, butylparaben standard references, each with a concentration of 0.05 mg/mL, and a standard solution of phenoxyethanol standard with a concentration of 0.2 mg/mL, using methanol as the solvent.

5.3.2. Mixed solution preparation

a. Mixed Standard Solution 1

The mixed standard solution was prepared and consisted PR and alpha-arbutin, a combined standard solution of PR and hydroquinone, a mixed standard solution of PR and kojic acid, a mixture of standard solutions of PR and vitamin C each with a concentration of 0.05 mg/mL. This mixed standard solution was used for the first specificity testing.

b. Mixed Standard Solution 2

A standard solution was made of a mixture of PR, methylparaben, ethylparaben, propylparaben, butylparaben standard with a concentration of 0.05 mg/mL and phenoxyethanol standard solution with a concentration of 0.02 mg/mL, using methanol. The standard solution of this mixture was used for HPLC system optimization and the second specificity testing.

5.3.3. Mixed solution preparation

As the cream matrices, the whitening cream formulation was composed with the ingredient as follows (the percentage is in w/v):

Stearate acid   10.0 %
Cetyl alcohol   1.5 %
Triethanolamine (TEA)   0.67 %
Propylene glycol   1.5 %
Isopropyl myristate   5.0 %
Glycerin   1.90 %
Disodium EDTA   0.40 %
Aquadest   add 100 %

All components were mixed to be a basic cream sample.

5.3.4. HPLC system optimization

The system optimization was conducted by determining the wavelength, mobile phase, and flow rate for the Waters e2695 instrument.

a. Wavelength

The wavelength selection was attempted by experiment and referring to the reported λ max for PR alone that was ± 280 nm [13, 15, 17].

b. Mobile phase

The mobile phase composed of a mixture of acetonitrile, water, and methanol [14] in the various comparisons. The process of mixing the mobile phase is carried out in the HPLC system to obtain a comparison that is always constant.

c. Flow rate

Optimization of flow rates was carried out starting from 0.8, 1.0, and 1.2 mL/min based on technical experience on the instrument used. Next, the suitability system parameters were evaluated, involving the retention time, area RSD, the number of theoretical plates, tailing factors, capacity, and resolution [20-25].

5.3.5. Suitability system test

The suitability test was done by injecting the solution of PR with the five preservatives for six times. The measurement result was then evaluated against the parameters: resolution (RS) > 1.5; tailing factor (Tf) = 0.9-1.4; capacity factor (k >10); the number of theoretical plates (N)> 2000 and RSD peak area ≤ 2% [20-25].

a. Injection Precision

The sample was injected 6x an in 10 and 20 μL, and the relative standard validation was calculated following Equation 3 below:

$$RSD = \frac{SD}{x} \cdot 100\%$$

$$SD = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n-1}}$$

$$\bar{x} = \frac{\sum x_i}{n}$$

[Eq. 3]

The requirement mentioned for injection precision: RSD ≤ % 1, n > 5 [23,24].

b. Capacity factor

The capacity factor represents how many times the analyte is retained relative to an unretained component. It is equivalent to the ratio of the analyte's retention time to the elution time of an unretained compound. The calculation following the technical requirement which stated that k' > 2, following Equation 4 [20-25].

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$$k' = \frac{tr - t_0}{t_0}$$ [Eq. 4]

Note: k' is the capacity factor, tr is retention time, and t_0 is the time retention of an unretained compound.

c. Resolution

Resolution (Rs) is the degree of separation of the analyte peaks to the closest one and is defined as the difference in retention times of the two peaks divided by the average peak width. The requirement is: Rs > 1.5 [23-25]. R is a function of column efficiency, N, to establish the general resolving power of the system, calculated using Equation 5 below [20-25]:

$$R_s = \left(\frac{t_{R1} - t_{R2}}{(W_{b1} - W_{b2})/2}\right)^2 = \left(\frac{\Delta t_R}{W_b}\right)^2$$ [Eq. 5]

d. Tailing factor

Tailing factor (Tf) represents the integrity of a peak, with te standard requirement: Tf ≤ 1.5 [20-25]. It was calculated by the formula in Equation 6 as follows:

$$Tf = B/A$$ [Eq. 6]

Whereas Tf is tailing factor, B = distance from the point at peak midpoint to the trailing edge (measured at 10 % of peak height), and A = distance from the leading edge of a peak to the midpoint (measured at 10 % of peak height).

e. Theoretical Plate Number

Column efficiency, symbolized by N, which must be > 2000 was measured using the following equation which has been derived for Gaussian peaks with Equation 7 below [23-25]:

$$N = \left(\frac{t_R}{\sigma}\right)^2 \quad ; N = 16\left(\frac{t_R}{W_b}\right)^2$$ [Eq. 7]

5.3.6. Validation Methods

a. Specificity

The standard solutions, both the single and mixed, solvent, and cream matrice, were analyzed one by one, thoroughly. The acceptability criterion was determined by seeing if it no disturbance at the retention time to the main analyte, where Rs is > 1.5.

b. Linearity and Range

• Linearity

Linearity test was carried out using one series of PR standard solutions in concentrations of 20%, 40%, 60%, 80%, 100%, and 120% w/v. A linear equation was made related to the area (y) to the concentration of the test solution (x). The intercept (a) and slope (b) was calculated and determined to make the linear regression equation (y = bx + a). The coefficient regression is calculated by Equation 8, and the result must be ≥ 0.999 [23-25].

$$r = \frac{\left[\sum_{i}^{n}(x_i - \bar{x})(y_i - \bar{y})\right]/(n-1)}{\left[\sum_{i}^{n}(x_i - \bar{x})^2(y_i - \bar{y})^2\right]/(n-1)^2}$$

[Eq. 8]

• Range

Data that has been taken from linearity, precision, and accuracy test (lowest to highest concentration) was employed to determine the range capacity of the method. The range of measurement is between the limit

of quantification to the highest concentration, which still gives appropriate RSD. It can also be generated from the linear regression equation [23-25].

a. Determination of Detection Limits (LOD) and Quantization Limits (LOQ)

LOD and LOQ were calculated from the linearity equation. It was calculated statistically from the calibration curve. As explained in the Table 4, LOD = 3.3(Syx/b) and LOQ = 10(Syx/b), whereas Syx is the standard deviation of the response, and b is the slope of the calibration curve [23-30].

b. Accuracy

Accuracy parameters were determined by calculating the percent recovery from three concentration levels, namely 80%, 100%, and 120% w/v. Each concentration was measured in three repetitions [23-25].

c. Precision

Precision testing used the 100% w/v level measured in six repetitions. These were made on the same day for the intra-day precision and in the different days inter-day test. The results of the intra-day and inter-day parameters were evaluated by its standard deviation and relative standard deviation results. The RSD was calculated from Horwitz's trumpet equation and HorRat. Acceptability criteria, RSD <2% and HorRat ratio <2% [23-26].

d. Robustness

Besides the rate of injection optimization, the robustness also was done by testing a method by deliberately making small changes to the procedure and evaluating the effect on the test results [23-25, 27-29]. In this study, small changes were made to the wavelength. The sample was measured at wavelengths of 280 nm and 282 nm, and then the results were tested using t-student compared to the validated method. In addition, the robustness test also was performed by changing the column (Enduro, Japan), conccurently with changes the solvent source/vendor, and performed by another analyst. The first condition used Zorbax's column (USA) and solvents from Jt. Baker (USA). The second condition employed a different brand column (Enduro, Japan) with the Merck-Sigma solvents, and the third condition utilized Enduro column and Jt. Baker's solvents. The robustness was evaluated the yield and variance of %-recovery, time of retention, resolution, and tailing factor [27-29].

5.3.7. Determination of the level of products on the market

The determination of three brands of whitening cream (namely as product A, B, and C) was carried out. The sample was prepared in a similar way with the self formulated cream *(Subsequence 5.3.3).* The amount of 1.0 g of the cream product was filled into a suitable beaker glass, added by 30 mL of solvent, and stirring homogeneously. The solution then was transferred to a volume flask of 50 mL, plus 1 mL of H_2SO_4 2 M, next was vortexed 5 min. Finally, the solution was added with methanol until the mark and shook. Afterward, it was filtered using a 0.20 μm membrane filter to be used as the HPLC test sample.

5.3.8. Sample preparation

The whitening cream from the market and a self-formulated sample was weighed as 1.0 g and filled into a suitable beaker glass, added 30 mL of solvent, and stirring homogeneously. It was then transferred to a volume flask of 50 mL, plus 1 mL of H_2SO_4 2 M, next was vortexed 5 min. Finally, the solution was added with methanol until the mark and shook. Afterward, it was filtered using a 0.20 μm membrane filter to be used as the HPLC test sample.

Appendix A. Supplementary Material

Supplementary material related to this article can be accessed at http://doi.org/10.35333/jrp.2020.228.

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